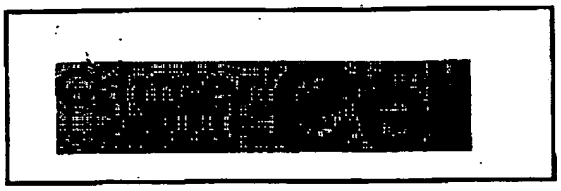


Cancer Cell, Vol 1, 325-337, May 2002

Selectively replicating adenoviruses targeting deregulated E2F activity are potent, systemic antitumor agents

Leisa Johnson [14], Annie Shen [1], Larry Boyle [1], John Kunich [1], Kusum Pandey, Marilyn Lemmon [1], Terry Hermiston [16], Marty Giedlin [17], Frank McCormick [2] and Ali Fattaey [15]

[1]Onyx Pharmaceuticals, Richmond, CA 94806 USA

[2]UCSF Comprehensive Cancer Center, San Francisco, CA 94115 USA

Correspondence:
Leisa Johnson
1 650 837 7170 (phone)
1 650 837 7240 (fax)
ljohnson@exelixis.com

Summary

Summary Significance Introduction Results Discussion Experimental procedures Referenc

We have engineered a human adenovirus, ONYX-411, that selectively replicates in human tumor cel but not normal cells, depending upon the status of their retinoblastoma tumor suppressor protein (pR pathway. Early and late viral gene expression as well as DNA replication were significantly reduced functional pRB-pathway-dependent manner, resulting in a restricted replication profile similar to tha nonreplicating adenoviruses in normal cells both in vitro and in vivo. In contrast, the viral life cycle tumor cell killing activity of ONYX-411 was comparable to that of wild-type adenovirus following infection of human tumor cells in vitro as well as after systemic administration in tumor-bearing animals.

Significance

Summary Significance Introduction Results Discussion Experimental procedures Referenc

Therapeutic agents with enhanced efficacy and strong tumor-cell selectivity are needed to treat huma cancers. One novel strategy is to use selectively replicating human adenoviruses, which amplify and spread throughout the tumor tissue, resulting in direct lysis of an expanded population of cancer cells We have engineered ONYX-411 to take advantage of the disruption of the pRB-pathway, one of the hallmarks of human cancer. Because of its strict dependency upon the functional status of the pRB-pathway, ONYX-411 has a high therapeutic index and is effective as a systemic antitumor agent in animal models of human cancer. In addition, ONYX-411 gene expression is highly tumor cell selecti making it an ideal delivery vehicle that will express therapeutic transgenes specifically at the tumor s

Introduction

Summary Significance Introduction Results Discussion Experimental procedures Referenc

Human adenoviruses normally infect, propagate, and lyse their host cells to release newly generated progeny virus. Although these properties are ideal for killing human cancer cells, virus replication m be restricted to tumor and not normal cells and tissues. Mutant adenoviruses have now been develop that are restricted from efficiently completing their life cycle in normal cells, but selectively propaga in and lyse tumor cells. In multiple clinical trials and animal models of human cancer, selectively replicating adenoviruses have shown varying degrees of antitumor efficacy.

Different approaches have been taken to restrict human adenovirus replication in normal cells (Kirn al., 2001; McCormick, 2001). Originally, the recognition that both tumor cells and human adenoviruses inactivate the p53 and pRB tumor suppressor protein functions led to the strategy for engineering adenoviruses to selectively propagate in human cancer cells based upon their p53- or pR pathway status (Bischoff et al., 1996; Fueyo et al., 2000). This strategy is based upon the concep that the loss of pRB- or p53-pathway functions in tumor cells should permit replication of E1A or E1 gene mutant viruses, respectively (Bischoff et al., 1996; McCormick, 2001).

Following adenovirus infection, E1A is the first viral gene product expressed, and is required for efficient transcription of all other viral early gene regions (Berk, 1986; Berk et al., 1979; Jones a Shenk, 1979). E1A also promotes S phase entry, partly through binding and inactivation of pRB ar its related family members (Bayley and Mymryk, 1994; Howe and Bayley, 1992; Howe et al., 1990; Wang et al., 1991, 1993). The adenovirus E1B region encodes two major polypeptides, E1 19K and E1B-55K, both of which function to inhibit p53-mediated apoptosis of virally infected cells (Debbas and White, 1993; Lowe and Ruley, 1993; White, 1995). E1B-55K directly binds to p and represses its transactivation activity (Teodoro and Branton, 1997; Teodoro et al., 1994; Yew and Berk, 1992; Yew et al., 1994), and is required in complex with the adenovirus E4 gene regic product, E4orf6, to target p53 for degradation (Moore et al., 1996; Nevels et al., 1997; Querido e al., 1997; Steegenga et al., 1998).

Like E1A and E1B, the adenoviral E4 gene region is expressed early after infection and is an essenti component of the viral life cycle. The E4 gene region encodes several products whose functions affe the nucleocytoplasmic shuttling of viral RNAs, efficient synthesis and processing of early and late vi proteins, the induction of apoptosis, and cellular transformation. The E4 gene products function in concert with E1A and E1B to create a cellular environment permissive for efficient expression and

processing of viral gene products and ultimately a productive virus infection.

ONYX-015 (*dl*1520), an E1B-55K gene-deleted human adenovirus that cannot inactivate p53 (Barker and Berk, 1987), was the first therapeutic virus shown to preferentially propagate in cancer cells based upon their p53 functional status (Bischoff et al., 1996; Ries et al., 2000; Yang et al., 2001). ONYX-015 is currently in phase III clinical trials in combination with standard chemotherapy for the treatment of patients with recurrent cancers of the head and neck.

pRB plays an essential regulatory role in mediating appropriate cellular responses to extracellular stimuli (Bartek et al., 1997; Larminie et al., 1998; Weinberg, 1995). pRB functions as a transcriptional repressor to negatively regulate S phase entry by directly binding to and repressing the transactivation activity of the E2F family of transcription factors (Dyson, 1998; Harbour and Dean, 2000). By binding to their promoters, E2Fs regulate the expression of a diverse set of genes involved in nucleotide metabolism, DNA replication, cell cycle control, apoptosis, and differentiation (Helin, 1998; Johnson and Schneider-Broussard, 1998; Muller and Helin, 2000). DNA tumor viruses target pRB family members, resulting in elevated E2F activity and efficient S phase entry, the cell cycle phase most permissive for viral DNA replication and progeny production (Dyson et al., 1989; Moran, 1993). For human adenovirus, two noncontiguous domains of E1A, conserved region 1 (CR1, low affinity) and conserved region 2 (CR2, high affinity), are required to mediate pRB binding and inactivation (Dyson et al., 1992; Fattaey et al., 1993). In concert with E1A function, human adenoviruses further target and direct E2F onto the viral E2 promoter through direct complex formation between the E4 gene region product, E4orf6/7, and E2F (Cress and Nevins, 1994; Helin and Harlow, 1994; Neill et al., 1990; Neill and Nevins, 1991; Obert et al., 1994; Raychaudhuri et al., 1990).

Human adenoviruses carrying mutations within the E1A-CR2 domain have been tested in the laboratory as potential anticancer agents (Fueyo et al., 2000; Heise et al., 2000). Although these virus mutants can efficiently replicate in both dividing and nondividing tumor cells, their selectivity in normal cells and safety in animal models has not been demonstrated. Another adenovirus mutant has recently been described that targets both the p53 and pRB pathways (Ramachandra et al., 2001). Similarly, the mechanism providing its tumor selectivity or in vivo utility as a safe and effective systemic anticancer agent has yet to be demonstrated.

An alternative approach for developing tumor cell selectivity has been the use of tissue- or tumor-specific promoters to control the expression of the adenovirus early gene regions E1A, E1B, E2, or E4 (Brunori et al., 2001; Doronin et al., 2001; Fuerer and Iggo, 2002; Hallenbeck et al., 1999; Hernandez-Alcoceba et al., 2000; Kurihara et al., 2000; Rodriguez et al., 1997; Yu et al., 1999a, 1999b). By definition, tissue-specific promoters lack tumor cell selectivity. Another limitation of this approach is that the selected promoter may not be active in all tumor cells derived from the particular tissue type for which it was chosen. This may be due to heterogeneity in the genetic alterations and differentiated state of many tumors, both of which could influence the activity of tissue-specific promoters. Furthermore, the mechanism used to establish selectivity may limit their broad utility to treat cancers of different tissue types.

Here we present a safe and systemically effective human adenovirus, ONYX-411, that selectively

replicates in a broad array of human tumor cell types based upon their defective pRB-pathway status. The pRB-pathway is disrupted in nearly all human cancers (Hanahan and Weinberg, 2000; Sherr, 2000), and hence, the oncolytic activity of ONYX-411 is not limited to tumors of a particular tissue type. ONYX-411 contains a deletion within the E1A-CR2 region, and its pRB-selectivity was further enhanced by replacement of the viral E1A and E4 promoter regions with the human E2F1 gene promoter. The combination of these attributes was essential for selective viral gene expression, viral DNA replication, and tumor cell killing both in vitro and in vivo.

Results

Summary Significance Introduction Results Discussion Experimental procedures References

Construction and characterization of virus mutants

Our goal was to develop a human adenovirus that can selectively replicate in human cancer cells based upon disrupted pRB-pathway function. In order to generate pRB-pathway selectivity, we chose to regulate the expression of the E1A and E4 gene products using the pRB-responsive human E2F1 promoter. This promoter was selected because: (1) it is subject to autoregulatory control in a cell cycle-dependent manner via four consensus E2F DNA binding sites; (2) its activity closely correlates with the pRB-pathway status of the cell; (3) the E2F DNA binding sequences present within the promoter mediate both repression by pRB and stimulation by wild-type, but not CR2 mutants of E1A; and (4) it has been used to direct pRB-dependent, tumor-selective gene expression in vivo, which differentiates between normal and aberrant cell cycle states (Johnson et al., 1994; Neuman et al., 1994; Parr et al., 1997).

To test pRB-responsiveness, we constructed plasmids representing the left end of the *dl*922/947 (E1A-ΔCR2) human adenovirus genome (Whyte et al., 1989) in which the E1A promoter region was replaced with the previously characterized wild-type or mutated alleles of the human E2F1 promoter (Parr et al., 1997) (Figures 1 and 2). We chose to use the *dl*922/947 mutation of E1A for our studies since it had been shown that E1A-ΔCR2 alleles were not able to overcome pRB-mediated repression of the E2F DNA binding sequences present within this promoter. In addition, human adenoviruses carrying mutations within this E1A domain have previously been tested for their selective replication properties in cancer cells (Fueyo et al., 2000; Heise et al., 2000). To demonstrate pRB-regulated E1A gene expression, C33-A cervical carcinoma cells (pRB mutant) were cotransfected with these constructs along with expression vectors containing either wild-type pRB or pRB-ΔCdk, an allele of pRB mutated in all potential CDK phosphorylation sites (Leng et al., 1997). pRB-ΔCdk is a constitutively active allele of pRB that is not subject to cell cycle regulation. This allele was used to distinguish between direct inactivation of pRB by E1A versus inactivation of pRB by Cdk-mediated phosphorylation as a consequence of E1A-induced S phase entry. Ectopic expression of either pRB or pRB-ΔCdk resulted in significant repression of the human E2F1 promoter and E1A expression (Figure 2A, lanes 2 and 3). Importantly, pRB-mediated repression was dependent upon intact E2F DNA binding sequences, as the promoter construct mutated only in the E2F DNA binding sites was insensitive to pRB expression (Figure 2A, lanes 5 and 6). Interestingly, the highest levels of expression were also observed when E1A was regulated by the wild-type E2F1 promoter (Figure 2A, compare lane 1 to lanes 4 and 7). This was in accordance with previous observations regarding the relative strength of the E2F1 promoter (Parr et al.,

1997). It is important to note that the endogenous E1A promoter was also responsive to pRB-ΔCdk expression (Figure 2A, lane 9). This may be due to either direct regulation by pRB through the E2F DNA binding sites in the endogenous E1A promoter or as a consequence of cell cycle arrest induced by pRB-ΔCdk expression.

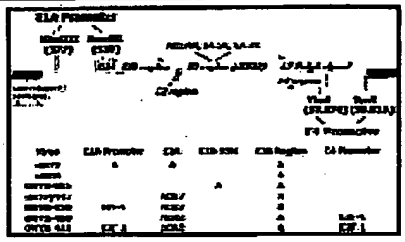


Figure 1. Diagram of viruses examined throughout the course of this study

Mutations specific to each virus are indicated by the affected region. The presence of a complete deletion is represented by Δ, whereas the minimal deletion of the E1A-CR2 domain from nucleotides 922 to 947 is indicated by ΔCR2. All viruses were created on an Ad5, *dl*309 background, which is characterized by a deletion within the E3B region that removes the E3-10.4K, 14.5K, and 14.7K immunoregulatory genes. ONYX-015 (*dl*1520) carries a deletion of the E1B-55K gene, whereas *dl*922/47 contains the minimal E1A-CR2 deletion. Unique restriction sites were engineered into the *dl*922/47 backbone such that the E1A and/or E4 promoters could be selectively replaced with the human E2F1 promoter. In the case of the E1A promoter, HindIII and BamHI sites were generated at nucleotides 377 and 538, respectively. The E4 promoter was replaced through the use of newly created XhoI and SpeI sites at nucleotides 35,576 and 35,815, respectively. Importantly, core enhancer and DNA packaging elements remained intact at both ends of the viral genome.

View larger version: [In this window] [In new window]

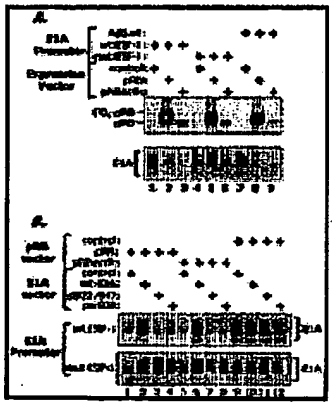


Figure 2. The human E2F1 promoter is responsive to the status of pRB and E1A

A: The E1A promoter was either left intact (Ad5) or replaced with the human E2F1 promoter, wild-type (wt) or mutated (mut) in the E2F/DP binding sites, in a plasmid containing nucleotides 22 to 5,790 of the Ad5 genome. Each plasmid carried an additional deletion within the E1A-CR2 domain from nucleotides 922 to 947. The promoter-specific plasmids were examined for pRB-responsiveness by cotransfection assays with a vector expressing wt pRB or pRB-ΔCdk in C33-A cells, a tumor cell line that is deleted for *RB1*. Lysates were harvested 48 hr posttransfection and analyzed for pRB and E1A expression by immunoblotting. **B:** The same plasmids in **A** were cotransfected with an additional plasmid that constitutively expressed a truncated version of E1A that was wt, deleted (*dl*922/947), or mutated (*pm*928) within the E1A-CR2 domain. These constitutively expressed forms were truncated at their C termini so that they could be distinguished from the reporter, E2F1 promoter-driven E1A. Lysates were harvested and analyzed for reporter-specific E1A expression as in **A**. The results are representative of two independent experiments.

View larger version: [In this window] [In new window]

These results demonstrated pRB-mediated regulation of the E2F1 promoter in the context of the adenoviral genome as measured by E1A expression. We chose to use an E1A-ΔCR2 allele for our studies since it is possible that any residual wild-type E1A expression could inactivate pRB, thereby circumventing regulation of the E2F1 promoter. To demonstrate this possibility, experiments similar to those in Figure 2A were conducted, except that an additional plasmid was cotransfected that contained either wild-type E1A or E1A-ΔCR2 mutant alleles (*dl*922/947 or pm928$^{C\rightarrow G}$). Expression of exogenous wild-type E1A (Figure 2B, lanes 2 and 6), but not E1A-ΔCR2 mutants (Figure 2B, lanes 3, 4, 7, and 8), was capable of inactivating pRB- and pRB-ΔCdk-mediated repression of the E2F1 promoter. Moreover, this inactivation by wild-type E1A was dependent upon intact E2F DNA binding sites within the E2F1 promoter. These results demonstrated that: (1) the E2F1 promoter was active in the context of the left end of the adenoviral genome, and (2) its activity was responsive to pRB-pathway status only in combination with ΔCR2 mutant alleles and not wild-type E1A. Based on these results, we modified the E1A-ΔCR2 mutant adenovirus, *dl*922/947 (Whyte et al., 1989), by replacing viral regulatory sequences within the E1A (ONYX-150), E4 (ONYX-410), or both gene regions (ONYX-411) with the human E2F1 promoter (Figure 1).

Viral gene expression, induction of S phase entry, and viral DNA replication

To examine the link between *RB* status and viral gene expression, primary mouse embryonic fibroblasts (MEFs) from isogenically matched $RB^{+/+}$ and $RB^{-/-}$ littermates were infected with the panel of adenoviruses shown in Figure 1. Regulation of the E1A region (ONYX-150) of human adenovirus by the E2F1 promoter resulted in a partial repression of E1A protein expression in $RB^{+/+}$ but not $RB^{-/-}$ MEFs (Figure 3). The most significant reduction in E1A expression levels, however, was observed when the E2F1 promoter regulated both the E1A and E4 regions (ONYX-411). This reduction in E1A levels was only observed in the $RB^{+/+}$ and not the $RB^{-/-}$ MEFs and was independent of their proliferation status (Figure 3, lanes 17–21). Interestingly, elevated E1A levels were observed in MEFs infected with ONYX-410 (see Discussion). These results indicate that pRB-regulated E1A gene expression was best achieved with ONYX-411, where the E2F1 promoter controlled both the E1A and E4 gene regions.

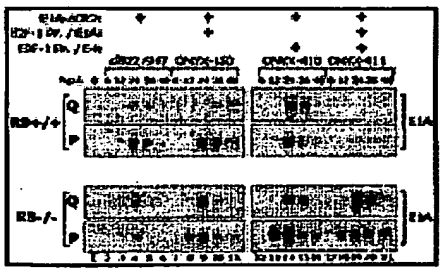


Figure 3. E2F1 promoter-regulated adenoviruses are pRB-responsive

Primary mouse embryonic fibroblasts (MEFs) from isogenically matched $RB^{+/+}$ and $RB^{-/-}$ littermates were propagated under proliferating (P) or quiescent (Q) growth conditions and infected with *dl*922/47, ONYX-150, ONYX-410, or ONYX-411. A "+" sign indicates the alterations specific to each virus. Cell lysates were generated at various hours postinfection (hpi) and examined

for E1A protein expression by immunoblot analysis. The results are representative of two independent experiments.

View larger version: [In this window] [In new window]

E2F activity is deregulated in most human cancers as a result of pRB-pathway disruption. Regulated expression of the E1A and E4 gene regions by the E2F1 promoter should, therefore, be preferentially restricted to human tumor cells and not normal cells. To test this directly, early and late viral protein expression was measured following infection of a panel of human tumor cell lines and normal proliferating (SAEC-P) or contact inhibited (SAEC-CI) primary human lung epithelial cells. We examined six different tumor cell lines of lung (H1299, H2009, H460, and H2172), pancreatic (MiaPaCa), and breast (MDA-MB-231) tissue origin, all of which were defective in their pRB-pathway status (Figures 4A–4C and data not shown; see Experimental Procedures for the status of pRB-pathway components in each cell line). E1A protein levels were significantly lower in primary human lung epithelial cells (SAEC-P and SAEC-CI) infected with either ONYX-150 or ONYX-411, where E1A was regulated by the human E2F1 promoter (Figure 4A). This was in contrast to the E1A levels detected in normal cells following infection with all of the other viruses where E1A was controlled by its endogenous promoter (*dl*309, *dl*922/947, ONYX-015, and ONYX-410) (Figure 4A). Importantly, E1A expression was at wild-type or enhanced levels in tumor cells infected with all viruses (Figure 4A). Similar to results obtained after infection of MEFs, E1A expression was markedly higher and sustained following infection of primary human cells with ONYX-410 (see Discussion). Expression of the E4 gene region products, E4orf6 and E4orf6/7, was significantly reduced in normal cells infected with ONYX-410 and ONYX-411, where E4 transcription was controlled by the human E2F1 promoter (Figure 4B). This was in contrast to the near wild-type expression kinetics and levels of E4orf6/7 detected in tumor cells infected with all viruses (Figure 4B). We were unable to readily detect E4orf6 expression in infected tumor cells in these studies. Taken together, the results in Figures 4A and 4B indicate that E2F1 promoter-regulated transcription of the E1A and E4 viral gene regions resulted in a significant repression of early viral gene expression in normal versus tumor cells.

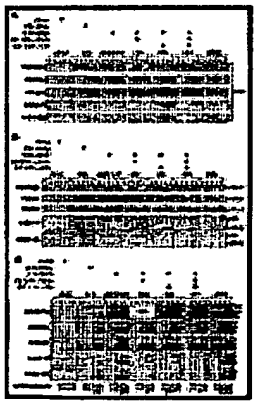


Figure 4. E2F1 promoter-driven adenoviruses demonstrate tumor-selective gene expression in infected human cells

Human tumor cell lines (MiaPaCa, MB231, H1299) or primary small airway epithelial cells that were either proliferating (SAEC-P) or arrested through contact inhibition (SAEC-CI) were infected with *dl*312, *dl*309, ONYX-015, *dl*922/47, ONYX-150, ONYX-410, or ONYX-411. A "+" sign indicates the alterations specific to each virus. At various times postinfection (T1–T5), cell lysates were generated and analyzed by immunoblotting for their early and late gene expression profiles. The hours postinfection (hpi) represented by T1–T5 were as follows: MiaPaCa (14, 24, 36, 50, and 64 hpi), MB231 (12, 25, 36, 47, and 73 hpi), H1299 (12, 25, 37, 48, and 61 hpi), and SAEC-P/-CI (6, 12, 24, 36, and 48 hpi). **A–C:** Early gene expression patterns are demonstrated for E1A (A),

E4orf6 (**B**), and E4orf6/7 (**B**). In **C**, late gene expression patterns are shown for hexon (h), penton (p), and fiber (f). In all cases, equal loading was demonstrated by immunoblot analysis for β-actin (data not shown). In addition, we performed quantitative PCR analysis to examine adenovirus DNA levels at 12, 24, 36, and 48 hpi in SAEC-CI cells. Samples were analyzed in duplicate and are represented as the fold increase in signal relative to that detected with *dl*312 at 12 hpi. ND, not determined. These results are representative of at least two independent experiments in the primary SAECs and once in 6 different human tumor cell lines, three of which are shown.

View larger version: [In this window] [In new window]

Adenoviral early gene expression is required for induction of cellular S phase entry, viral DNA replication, and subsequent expression of late viral messages and capsid proteins. Therefore, we next examined how each of these steps was affected in the different E2F1 promoter-regulated viruses. To examine how viral early gene expression affects the host cell's division cycle, we analyzed the cell cycle profile of synchronized, contact-inhibited SAECs following infection with the panel of viruses used above. Cells were harvested at various times postinfection and analyzed by flow cytometry to determine the fraction of cells in the G1, S, or G2/M phases of the cell cycle. As compared to wild-type virus (*dl*309) infected cells, a significantly lower fraction of normal cells infected with ONYX-150, ONYX-410, or ONYX-411 were in S phase at various times postinfection (Figure 5). In contrast, the fractions of ONYX-015- and *dl*922/947-infected cells in S phase were similar to wild-type-infected cells (Figure 5). As determined by quantitative PCR analysis, the levels of viral DNA replication closely reflected both the time of entry and fraction of cells in S phase following infection with each of the viruses (Figure 4C). These studies demonstrate that ONYX-411 (regulation of both E1A and E4 gene regions by the E2F1 promoter) was severely attenuated in its ability to induce S phase entry and to replicate its DNA in normal cells.

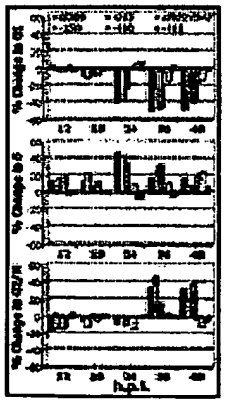


Figure 5. E2F1 promoter-driven adenoviruses are restricted in their ability to promote S phase entry

Primary human small airway epithelial cells (SAECs) were arrested by prolonged contact inhibition and infected with the indicated viruses. Cells were collected at various hpi and analyzed by flow cytometry. At a given time postinfection, the percentage change in the G1, S, and G2/M phases of the cell cycle that resulted following infection with each of the replication-competent adenoviruses is represented relative to the percentage of each phase following infection with the replication-deficient, *dl*312 adenovirus (e.g., % S phase [dl309–dl312] at equivalent hpi). These results are representative of at least two independent experiments.

View larger version: [In this window] [In new window]

Adenovirus late protein expression is dependent upon viral DNA replication. Therefore, we expected that the attenuated replication of the E2F1 promoter-regulated viruses in normal cells would result in decreased late viral protein expression. As compared to infected tumor cells, remarkably low levels of the viral capsid proteins hexon (h), penton (p), and fiber (f) were detected in contact inhibited normal lung epithelial cells infected with ONYX-150, ONYX-410, and ONYX-411 (Figure 4C). Although at lower than wild-type levels, capsid protein expression was observed in proliferating normal cells (SAEC-P, Figure 4C) infected with ONYX-150 and ONYX-410 (E2F1 promoter driving E1A or E4, respectively). Taken together, these results indicate that the combined regulation of the E1A and E4 gene regions (ONYX-411) resulted in the greatest inhibition of both cellular and adenoviral DNA replication as well as late viral protein expression in normal human cells, regardless of their proliferative status (Figures 4C and 5, and data not shown).

In vitro and in vivo efficacy of pRB-regulated adenoviruses

We next sought to determine the cell killing properties of the pRB-dependent viruses in infected normal and tumor cells. A quantitative cell viability assay was used to measure the extent of cell killing by each virus (see Experimental Procedures). The cell lines examined were selected to represent a broad range of tumor tissue types including carcinomas of the lung (H1299, H2009, H460, H522, H520, and A549), colon (SW620), pancreas (MiaPaCa), prostate (DU145), breast (MB231, MB468), cervix (C33-A), and head and neck (HLaC), as well as osteosarcomas (U2OS). The normal cells tested also represented a broad range of primary epithelial cell types, including lung (SAEC, small airway; or BEC, bronchial epithelial cells), liver hepatocytes, prostate (PrEC), breast (MEC), and kidney (REC), as well as endothelial cells (MVEC, microvascular endothelial cells derived from either lung or dermis). In the panel of tumor cell lines examined, all of the E2F1 promoter-driven viruses demonstrated a cell killing capacity that was comparable to wild-type adenovirus (Figure 6 and data not shown). In contrast, the E2F1 promoter-driven viruses were markedly attenuated in their ability to kill all normal cells tested, independent of the cellular proliferation status. ONYX-411's cytolytic activity in normal cells was comparable to the replication-defective adenovirus, *dl*312, and was approximately 100- to >1000-fold lower than wild-type adenovirus, *dl*309. These results demonstrate that ONYX-411 had the greatest degree of selective cell killing activity.

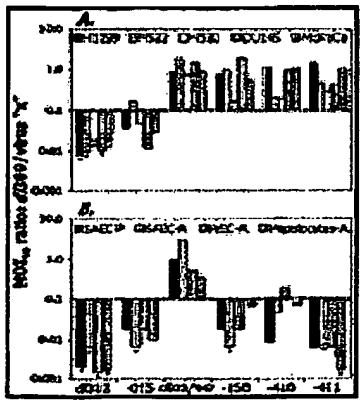


Figure 6. Selective tumor cell killing by E2F1 promoter-regulated adenoviruses

Cells were plated at varying densities and infected the following day at varying MOIs with the indicated viruses. **A and B:** At selected days postinfection, cell viability was determined by MTT analysis (see Experimental Procedures). For each tumor cell line and normal cell type, a minimum of two independent assays was each performed in quadruplicate. A representative subset of this data is shown for three human, matched tissue types of tumor (**A**) and normal (**B**) cell origin. For lung, 3 non-small cell lung carcinomas (H1299, H522, and H520) versus normal proliferating (SAEC-P) and contact inhibited (SAEC-A)

small airway epithelial cells are shown. The DU145 prostate carcinoma versus its normal, arrested epithelial cell counterpart, PrEC-A, is shown. Normal, arrested human hepatocytes were used as a tissue comparison for the pancreatic carcinoma cell line, MiaPaCa. MOI_{50} is defined as that MOI which results in 50% cell killing at a particular stop point. The data is presented as the MOI_{50} for each virus relative to that of *dl*309, where low MOI_{50} ratios are indicative of attenuation. In some instances (*), the degree of attenuation in cell killing by certain viruses is so great that the true MOI_{50} can not be determined. Therefore, in these cases, the extent of attenuation is actually greater than depicted. ND, not determined.

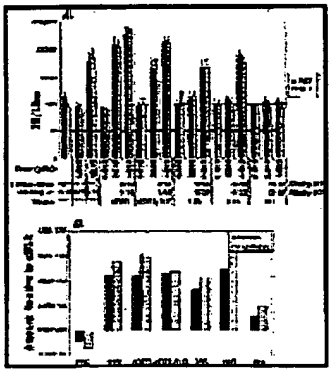
View larger version: [In this window] [In new window]

The systemic delivery of replicating human adenoviruses, even those modified for tumor-selective replication, has previously been shown to cause dose-limiting toxicity in mice. This toxicity is associated with liver necrosis and is thought to be a result of the administered dose of adenoviral coat proteins, viral early gene expression, viral replication, and/or de novo late gene expression. The host immune response may also play a role in mediating this dose-limiting toxicity. To evaluate the safety profile of the E2F1 promoter-driven viruses in vivo, the maximum tolerated single dose of each virus was determined following intravenous (i.v.) administration in Balb/c mice. Survival, weight loss, and serum levels of the liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were used as measures of toxicity following administration of increasing doses of virus.

One hundred percent survival and no increases in serum AST/ALT levels were observed in all of the vehicle control and experimental treatment groups (n = 10/cohort) at the 6×10^8 plaque forming unit (pfu) dose 48 hr (day 3) after virus administration (Figure 7A). Toxicities were first observed at the 2×10^9 pfu dose. Weight loss preceded death, which occurred as early as day 4 following wild-type (*dl*309) virus administration. At this dose, only 40% of the mice treated with wild-type virus survived to day 7, 30% survived to day 12 following ONYX-150 administration, and 80% survived to day 7 following ONYX-410 dosing. In contrast, all mice survived when treated at the 2×10^9 dose of either *dl*922/947 or ONYX-411 for the duration of the study (n = 10/cohort; data not shown).

Figure 7. ONYX-411 results in reduced systemic toxicity and increased survival following intravenous (i.v.) administration

A: BALB/c mice were injected with a single i.v. dose of increasing amounts (pfu) of the indicated viruses. On day 3, 48 hpi, mice (n = 3) were analyzed for their serum AST/ALT enzyme levels. Two independent studies were performed at the 4×10^9 dose, with the number of associated deaths at day 7 indicated. ND, not determined. **B:** Livers were harvested



on day 3 from mice (n = 3) injected at the 4×10^9 dose and analyzed for both adenoviral DNA and E1A-13S mRNA levels by quantitative PCR analysis. The resulting signals are represented relative to that detected with *dl*312-injected mouse liver samples (e.g., *dl*309 DNA signal/*dl*312 DNA signal).

View larger version: [In this window] [In new window]

At the single i.v. dose of 4×10^9 pfu, ONYX-411 was the only replicating virus that did not result in increased serum AST/ALT levels on day 3 (Figure 7A). At this dose, no mice survived beyond day 7 when treated with either wild-type or ONYX-410, and only one of twenty *dl*922/947-treated animals survived beyond day 7 (Figure 7A). In contrast, the ONYX-411 treated cohorts demonstrated a delayed toxicity beginning on day 8. ONYX-411 treated animals did not survive beyond day 12 in study 1, whereas all of the ONYX-411 treated mice survived to day 18 (duration of study) in the second study. Additional studies demonstrated that the ONYX-411-induced toxicity was always significantly lower in both the time of onset and the number of affected animals as compared with all other replicating viruses tested.

Adenovirus preparations often contain a substantial proportion of defective particles. It is important to note that the lower toxicities observed with the pRB-regulated viruses were not due to the administration of reduced virus particle numbers as the particle:pfu ratios for all viruses tested were within ~0.5- to 8-fold of each other. In fact, more particles per dose were delivered for all of the pRB-regulated viruses compared to wild-type virus, *dl*309 (data not shown). Therefore, we sought to determine if the reduced toxicity inherent to ONYX-411 correlated with viral replication and gene expression levels. Livers were harvested from mice (n = 3) on day 3 following administration of a single i.v. dose of 4×10^9 pfu and analyzed by quantitative PCR. The extent of E1A gene expression as well as adenoviral DNA replication closely mirrored the observed increases in serum AST/ALT levels at equivalent times postinjection for each of the adenoviruses (compare Figures 7A and 7B, and data not shown). As compared to wild-type virus (*dl*309), ONYX-411 exhibited the greatest attenuation in viral DNA replication (~50-fold) and E1A gene expression (~100-fold) in normal mouse livers in vivo (Figure 7B). The improved safety profile of ONYX-411 was further demonstrated in primary murine hepatocytes propagated in vitro, where it was significantly impaired for cell killing (>10-fold) as well as E1A and late protein expression when compared to wild-type virus (data not shown).

Taken together, these results demonstrated that ONYX-411 had the greatest selectivity index in vitro as well as the best safety profile in vivo when compared to the other adenoviruses tested. We next

evaluated the systemic antitumor efficacy of ONYX-411 in nude mice (n = 10/cohort) bearing subcutaneous (s.c.) C33-A human xenograft tumors. A total dose of 2×10^9 pfu was administered intravenously as a daily regimen of 4×10^8 pfu for five consecutive days. Both ONYX-411 and wild-type virus (*dl*309) demonstrated a significant survival advantage relative to vehicle control ($p = 0.03$ and 0.005, respectively), and resulted in equivalent numbers of complete regressions (CRs: n = 3/10; Figure 8A). Similar levels of ONYX-411 and *dl*309 virus replication were observed in tumor samples by in situ hybridization at 60 and 66 days posttreatment, respectively (Figure 8B). In two independent studies using the C33-A cervical cancer xenograft model, increasing the dose of ONYX-411 by approximately 2-fold (3.75×10^9 pfu total, administered as 7.5×10^8 pfu on days 1–5) resulted in a comparable survival advantage at day 70 (range: 60%–70% of mice) without increased toxicity (Figure 8A). In these studies, cure rates ranged from 25%–60% (Figure 8A). Subsequent studies have also demonstrated that ONYX-411 can be administered systemically in tumor bearing, immunocompromised mice (Balb/c genetic background) at doses up to a total of 5×10^9 pfu (1×10^9 pfu administered on days 1–5) with no increased toxicity. This dose is 2-fold higher than the previously reported tolerated dose for ONYX-015 (Heise et al., 1999⊞) (data not shown).

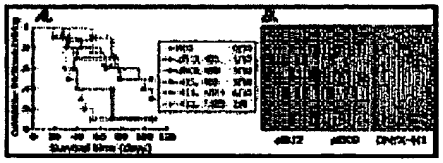


Figure 8. Tumor efficacy and evidence of viral replication following i.v. administration of ONYX-411 in mice bearing C33-A human tumor xenografts

A: Mice (n = 8 or 10) were treated with an i.v. injection dose of vehicle control (PBS) or either 2×10^9 pfu or 3.75×10^9 pfu of the indicated viruses administered as a daily regimen of 4×10^8 or 7.5×10^8 pfu, respectively, for five consecutive days on days 1 through 5 of the study. The cumulative fraction of mice surviving over time is plotted. The observed number of complete responses for each cohort is indicated in the legend immediately following the daily dose. The three ONYX-411 cohorts were from three independent studies; those viruses above the dotted line were all done within the same study, whereas the two cohorts of ONYX-411 below the dotted line were from two separate studies. **B:** Tumors were isolated from mice dosed at 2×10^9 pfu in **A** on day 71 (*dl*312 and *dl*309) and day 65 (ONYX-411) of the study and examined for the presence of newly replicated adenoviral DNA by in situ hybridization. Virally infected tumor cells (dark blue stain; black arrow) are evident throughout the sections from mice infected with either *dl*309 or ONYX-411.

View larger version: [In this window] [In new window]

Discussion

Summary Significance Introduction Results Discussion Experimental procedures References

The pRB-pathway is disrupted in nearly all human cancers (Hanahan and Weinberg, 2000⊞; Sherr, 2000⊞). A number of strategies have been employed to target defects in this pathway in the hope of developing novel cancer therapeutic agents. These include small molecule inhibitors of Cdk4 kinase enzyme, delivery of *p16INK4a* or *RB1* genes, inhibitors of E2F DNA binding or dimerization, and various antisense approaches targeting activated components of the pRB-pathway (Garrett and Fattaey, 1999⊞; Rao, 1996⊞). Genetically engineered herpes simplex virus mutants have also been designed to target alterations within the pRB-pathway, thereby promoting an enhanced tumor-restricted replication profile (Chase et al., 1998⊞; Chung et al., 1999⊞; Kramm et al., 1997⊞; Mineta et al., 1994, 1995⊞; Nakamura et al., 2001⊞). We have developed ONYX-411 as a novel cancer therapeutic agent based upon the known functions of the viral early gene products during the adenovirus life cycle and their complex interactions with the pRB tumor suppressor pathway. ONYX-411 is based upon the *dl*922/947 human adenovirus that contains a 24 base pair deletion in the conserved region 2 of E1A (Whyte et al., 1989⊞). In addition, expression of the viral E1A and E4 gene regions was brought under the control of the cellular E2F1 promoter. We have demonstrated that the combination of these features in ONYX-411 was crucial for selective viral gene expression, replication, and progeny production in tumor but not normal human cells. This resulted in the selective killing of tumor cells in vitro, reduced in vivo systemic toxicity, and a survival benefit in animal models of human cancer following systemic administration.

E1A-ΔCR2 mutant adenoviruses have previously been shown to possess antitumor activity in culture and in in vivo models of human cancer (Fueyo et al., 2000⊞; Heise et al., 2000⊞). Although our results are consistent with previous observations that E1A-ΔCR2 adenoviruses replicate efficiently in human tumor cells, we have not observed the level of selectivity with these viruses that is required for them to be considered tumor-specific and safe cancer therapeutic agents. This may partly be explained by the fact that while E1A-ΔCR2 mutant adenoviruses are compromised in their ability to inactivate pRB, they are still capable of mediating S phase entry via their intact CR1 domain (Howe et al., 1990⊞; Wang et al., 1991, 1993⊞). Furthermore, both early and late viral gene expression remain intact following infection with these viruses, even in normal human cells (see Figures 4A–4C).

We have shown that regulation of both the E1A and the E4 gene regions by the human E2F1 promoter is necessary to achieve pRB-pathway selectivity for replication and late viral gene expression. Although not tested in the context of the full viral genome in this study, we provide evidence that expression of wild-type E1A is able to overcome the pRB-regulated nature of the E2F1 promoter inserted in the left end of the adenovirus genome. This indicates that the selectivity provided by the E2F1 promoter for controlling viral gene expression can only be achieved in combination with mutations in the E1A-CR2 region. In addition to E1A, the E4orf6/7 gene product is known to independently enhance E2F activity on the adenovirus E2 promoter (Helin and Harlow, 1994⊞; Neill et al., 1990⊞; Neill and Nevins, 1991⊞; O'Connor and Hearing, 1994⊞; Obert et al., 1994⊞; Raychaudhuri et al., 1990⊞; Reichel et al., 1989⊞) as well as the cellular, human E2F1 promoter (Schaley et al., 2000⊞). To this end, we have generated and examined an ONYX-411 virus that also contained an inactivating mutation within the E4orf6/7 gene product, but we did not observe an increase in its relative tumor versus normal cell killing selectivity (data not shown). Therefore, modulating the expression of the E4 gene region by the human E2F1 promoter appears to be sufficient to regulate these functions of E4orf6/7.

Interestingly, we detected enhanced E1A protein levels following infection of either normal human or mouse cells with ONYX-410 (E1A-ΔCR2, E2F1 promoter controlling E4). This upregulation in E1A protein levels may be attributed to the reduced E4 gene expression resulting from E2F1 promoter regulation. One candidate E4 gene product mediating this effect may be E4orf4, which has previously been shown to modulate E1A function (and possibly levels) through its interactions with the protein phosphatase, PP-2A (Kleinberger and Shenk, 1993; Muller et al., 1992; Whalen et al., 1997). Alternatively, E4orf6 and possibly E4orf6/7, which play novel roles in downregulating E1A protein levels (L.J., F.M., and C. O'Shea, unpublished data), may also contribute to this phenotype.

We closely examined the virus life cycle in normal human cells following infection with the E2F1 promoter-driven adenoviruses reported in this study. Based upon our results with ONYX-150 and ONYX-411, it is clear that in order to mediate efficient S phase entry and viral DNA replication, the onset and expression of E1A must be near wild-type levels. In addition, as evident from cells infected with ONYX-410 and ONYX-411, robust E4 gene expression is also required for efficient viral DNA replication. Therefore, we conclude that the life cycle of the pRB-regulated viruses, and in particular ONYX-411, is halted prior to viral DNA replication in normal human cells. It should also be noted that while it is positively regulated by E1A, transcription of the E4 gene region can occur in an E1A-independent manner. This is evident from E4 gene expression at a time when E1A protein was not detectable in ONYX-150 infected normal human cells (Figure 4B).

ONYX-015 (*dl*1520) was the first adenovirus developed based upon its ability to selectively replicate in human cancers and has shown great promise in late-phase clinical trials (Khuri et al., 2000; Kirn, 2001; Nemunaitis et al., 2000, 2001a, 2001b). ONYX-015 replication is rendered p53-pathway selective by virtue of a deletion of the E1B-55K gene (Barker and Berk, 1987; Bischoff et al., 1996; Ries et al., 2000; Yang et al., 2001). In complex with E4orf6, E1B-55K not only mediates p53 degradation, but also plays an important role in regulating the nucleocytoplasmic shuttling of late viral messages and shutoff of host cell protein synthesis. Thus, deletion of the entire E1B-55K gene in ONYX-015 may contribute to its attenuated replication in some human cancer cell lines. In this respect, adenovirus mutants have recently been generated that specifically target E1B-55K's p53 binding function while preserving its role(s) late in the viral life cycle (Shen et al., 2001). In cell culture studies, these viruses demonstrated an improved replication capacity compared to that of ONYX-015. Similarly, in our in vitro studies, ONYX-411 demonstrated a higher cell killing capacity than ONYX-015 in nearly all cell lines examined.

A number of studies have previously characterized an inherent toxicity following intravenous administration of human adenoviruses in mice (Duncan et al., 1978; Engelhardt et al., 1994; Heise et al., 1999; Yang et al., 1994). Increased serum levels of the liver enzymes AST and ALT, degeneration of liver tissue, and severe weight loss often accompany these toxicities. Although the underlying mechanisms responsible for the lower systemic toxicity associated with ONYX-411 are not clearly resolved, the combination of all three features built into this virus contribute to this phenotype. *dl*922/947 (E1A-ΔCR2), ONYX-150 (E1A-ΔCR2, E2F1 promoter controlling E1A), and ONYX-410 (E1A-ΔCR2, E2F1 promoter controlling E4) all demonstrated significantly higher levels of E1A gene expression, DNA replication, and ensuing systemic toxicity than was observed with ONYX-411. It is

likely, therefore, that either E1A, certain E4 gene products, or the coincident expression of these gene products in infected mouse liver tissue contributes to the hepatic toxicity observed in animals treated with human adenoviruses. Tumor necrosis factor-α, TNF-α, is induced in response to viral infection, and it has been speculated that the combined action of E1A and TNF-α may also play a role in mediating this toxicity. Further analysis using an expanded panel of virus mutants in mice of different genetic backgrounds is required to fully understand the mechanisms of induced toxicity.

We have shown that when administered systemically, ONYX-411 has in vivo antitumor activity, resulting in significant survival benefits that are comparable to wild-type adenovirus. This property, combined with the significant selectivity for tumor versus normal tissues as demonstrated both in cell culture and by the reduced systemic toxicity in vivo, identifies ONYX-411 as a promising selective cancer therapeutic agent. Importantly, the E2F-dependent characteristics of ONYX-411 make it a broad-based cancer therapeutic, given that E2F activity is upregulated as a result of pRB-pathway alterations in nearly all human cancers. This is in stark contrast to many of the other viral-based cancer therapies to date, which exploit the use of tissue-specific promoters, thereby limiting the scope of their utility. Finally, in contrast to ONYX-015, the lack of viral gene expression exhibited by ONYX-411 in normal human cells (Figures 4A–4C) make it highly advantageous as a gene delivery vector for tumor-selective expression of transgenes with anticancer properties (Hawkins and Hermiston, 2001a, 2001b⊞; Hawkins et al., 2001⊞). Examination of ONYX-411's utilities for these strategies is currently underway.

Experimental procedures

Summary Significance Introduction Results Discussion Experimental procedures References

Transient transfections

C33-A cells were seeded into 10 cm dishes. The next day, 5 μg each of a promoter-specific reporter plasmid (pXC1.SV-922/947, pWT.E2F1P-922/947, or pMUT.E2F1P-922/947), a pRB expression plasmid (pCMV.Neo-Bam, pCMV.RB, or pCMV.RBΔcdk), and an E1A-ER expression plasmid (pCAN.myc1-ER, pCAN.E1A.WT-ER, pCAN.E1A.922/947-ER, or pCAN.E1A.928-ER) were mixed together with 10 μg of pGEM plasmid DNA (25 μg total DNA) and 250 mM $CaCl_2$. The DNA/$CaCl_2$ solution was then added dropwise, with constant bubbling, to an equal volume of 2× HBS (pH 7.25) and incubated for 25 min at RT. The DNA precipitate was then added slowly to the cells and left O/N at 37° C/5% CO_2. Transfections were stopped by washing the cell monolayer 2× with PBS/10% FBS, followed by replacement with growth medium supplemented with 10% FBS and 1 μM tamoxifin. Cells were harvested 48 hr posttransfection and lysates prepared as described below.

Flow cytometry analysis

Cells were washed 1× with PBS, harvested by trypsinization at 37°C for 5 min, neutralized in medium containing 10% FBS, and aggregates dispersed through repeated pipetting. Cells were then pelleted at 1,500 rpm, 4°C for 5 min, washed 1× with cold PBS/1% FBS and then 1× with cold PBS. Cell pellets were thoroughly resuspended in 200 μl of cold PBS and fixed O/N at 4°C following the slow addition of 3 mls of 70% EtOH/15 mM glycine (pH 2.8) while vortexing. Fixed cells were pelleted as before and washed 1× with cold PBS/1% FBS. Cell pellets were then resuspended in 500 μl of 1× PI buffer (PBS,

1% FBS, 250 μg/ml RNase A, and 15 μM propidium iodide), incubated for 30 min at 37°C, and either analyzed immediately or stored at 4°C in the dark for up to 24 hr. Samples were passed through a filter cap immediately prior to analysis on a FACSCalibur™, and data was analyzed either using ModFit or CellQuest.

Supplemental data

Additional experimental procedures can be found as supplemental data at http://www.cancercell.org/cgi/content/full/1/4/325/DC1.

Acknowledgments

We thank Sandy McCoy, Paul Harden, Adam Sampson-Johannes, Gabriel Brooks, Wen Yan, Amy Aspelund, Elizabeth Sevilla, Sarah White, Jenny Holt, and Lynda Hawkins for excellent technical support. We also thank Josh Watanabe, Adam Conner, Mebratu Gebreyesus, and Chris Bell for excellent assistance in virus production. For the animal studies, we thank Suzette Weber, Andrew Alva, Eric Suto, Eileen Szatkowski, Pia Roo, Joyce Conway, and Iris Albert for their tremendous technical support. We thank Clodagh O'Shea for helpful discussions and technical advice on FACS analysis as well as Gaston Habets for advice and assistance on TaqMan analysis. We thank Tyler Jacks, Kristian Helin, Wade Harper, Peter Whyte, Elizabeth Moran, Patrick Hearing, Eileen White, Ed Harlow, Tom Shenk, and Phil Branton for generously providing us with reagents, and Thomas Dubensky and Len Post for critical reading of the manuscript.

References

Summary Significance Introduction Results Discussion Experimental procedures References

⊞ Barker, D.D. and Berk, A.J. (1987). Adenovirus proteins from both E1B reading frames are required for transformation of rodent cells by viral infection and DNA transfection. Virology *156*, 107-121. [Medline]

⊞ Bartek, J., Bartkova, J., and Lukas, J. (1997). The retinoblastoma protein pathway in cell cycle control and cancer. Exp. Cell Res. *237*, 1-6. [Medline]

⊞ Bayley, S.T. and Mymryk, J.S. (1994). Adenovirus E1A proteins and transformation. Int. J. Oncol. *5*, 425-444. [Medline]

⊞ Berk, A.J. (1986). Functions of adenovirus E1A. Cancer Surv. *5*, 367-387. [Medline]

⊞ Berk, A.J., Lee, F., Harrison, T., Williams, J., and Sharp, P.A. (1979). Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs. Cell *17*, 935-944. [Medline]

⊞ Bischoff, J.R., Kirn, D.H., Williams, A., Heise, C., Horn, S., Muna, M., Ng, L., Nye, J.A., Sampson-Johannes, A., Fattaey, A., and McCormick, F. (1996). An adenovirus mutant that replicates selectively

in p53-deficient human tumor cells. Science *274*, 373-376. [Medline]

Brunori, M., Malerba, M., Kashiwazaki, H., and Iggo, R. (2001). Replicating adenoviruses that target tumors with constitutive activation of the wnt signaling pathway. J. Virol. *75*, 2857-2865. [Medline]

Chase, M., Chung, R.Y., and Chiocca, E.A. (1998). An oncolytic viral mutant that delivers the CYP2B1 transgene and augments cyclophosphamide chemotherapy. Nat. Biotechnol. *16*, 444-448. [Medline]

Chung, R.Y., Saeki, Y., and Chiocca, E.A. (1999). B-myb promoter retargeting of herpes simplex virus gamma34.5 gene- mediated virulence toward tumor and cycling cells. J. Virol. *73*, 7556-7564. [Medline]

Cress, W.D. and Nevins, J.R. (1994). Interacting domains of E2F1, DP1, and the adenovirus E4 protein. J. Virol. *68*, 4213-4219. [Medline]

Debbas, M. and White, E. (1993). Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. Genes Dev. *7*, 546-554. [Medline]

Doronin, K., Kuppuswamy, M., Toth, K., Tollefson, A.E., Krajcsi, P., Krougliak, V., and Wold, W.S. (2001). Tissue-specific, tumor-selective, replication-competent adenovirus vector for cancer gene therapy. J. Virol. *75*, 3314-3324. [Medline]

Duncan, S.J., Gordon, F.C., Gregory, D.W., McPhie, J.L., Postlethwaite, R., White, R., and Willcox, H.N. (1978). Infection of mouse liver by human adenovirus type 5. J. Gen. Virol. *40*, 45-61. [Medline]

Dyson, N. (1998). The regulation of E2F by pRB-family proteins. Genes Dev. *12*, 2245-2262. [Medline]

Dyson, N., Buchkovich, K., Whyte, P., and Harlow, E. (1989). Cellular proteins that are targetted by DNA tumor viruses for transformation. Princess Takamatsu Symp. *20*, 191-198. [Medline]

Dyson, N., Guida, P., McCall, C., and Harlow, E. (1992). Adenovirus E1A makes two distinct contacts with the retinoblastoma protein. J. Virol. *66*, 4606-4611. [Medline]

Engelhardt, J.F., Ye, X., Doranz, B., and Wilson, J.M. (1994). Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver. Proc. Natl. Acad. Sci. USA *91*, 6196-6200. [Medline]

Fattaey, A.R., Harlow, E., and Helin, K. (1993). Independent regions of adenovirus E1A are required for binding to and dissociation of E2F-protein complexes. Mol. Cell. Biol. *13*, 7267-7277. [Medline]

Fuerer, C. and Iggo, R. (2002). Adenoviruses with Tcf binding sites in multiple early promoters show enhanced selectivity for tumour cells with constitutive activation of the wnt signalling pathway. Gene Ther. *9*, 270-281. [Medline]

Fueyo, J., Gomez-Manzano, C., Alemany, R., Lee, P.S., McDonnell, T.J., Mitlianga, P., Shi, Y.X., Levin, V.A., Yung, W.K., and Kyritsis, A.P. (2000). A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect in vivo. Oncogene *19*, 2-12. [Medline]

Garrett, M.D. and Fattaey, A. (1999). CDK inhibition and cancer therapy. Curr. Opin. Genet. Dev. *9*, 104-111. [Medline]

Hallenbeck, P.L., Chang, Y.N., Hay, C., Golightly, D., Stewart, D., Lin, J., Phipps, S., and Chiang, Y.L. (1999). A novel tumor-specific replication-restricted adenoviral vector for gene therapy of hepatocellular carcinoma. Hum. Gene Ther. *10*, 1721-1733. [Medline]

Hanahan, D. and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70. [Medline] [Full Text]

Harbour, J.W. and Dean, D.C. (2000). The Rb/E2F pathway: expanding roles and emerging paradigms. Genes Dev. *14*, 2393-2409. [Medline]

Hawkins, L.K. and Hermiston, T. (2001). Gene delivery from the E3 region of replicating human adenovirus: evaluation of the E3B region. Gene Ther. *8*, 1142-1148. [Medline]

Hawkins, L.K. and Hermiston, T.W. (2001). Gene delivery from the E3 region of replicating human adenovirus: evaluation of the ADP region. Gene Ther. *8*, 1132-1141. [Medline]

Hawkins, L.K., Johnson, L., Bauzon, M., Nye, J.A., Castro, D., Kitzes, G.A., Young, M.D., Holt, J.K., Trown, P., and Hermiston, T.W. (2001). Gene delivery from the E3 region of replicating human adenovirus: evaluation of the 6.7 K/gp19 K region. Gene Ther. *8*, 1123-1131. [Medline]

Heise, C., Hermiston, T., Johnson, L., Brooks, G., Sampson-Johannes, A., Williams, A., Hawkins, L., and Kirn, D. (2000). An adenovirus E1A mutant that demonstrates potent and selective systemic anti-tumoral efficacy. Nat. Med. *6*, 1134-1139. [Medline]

Heise, C.C., Williams, A.M., Xue, S., Propst, M., and Kirn, D.H. (1999). Intravenous administration of ONYX-015, a selectively replicating adenovirus, induces antitumoral efficacy. Cancer Res. *59*, 2623-2628. [Medline]

Helin, K. (1998). Regulation of cell proliferation by the E2F transcription factors. Curr. Opin. Genet. Dev. *8*, 28-35. [Medline]

Helin, K. and Harlow, E. (1994). Heterodimerization of the transcription factors E2F–1 and DP-1 is required for binding to the adenovirus E4 (ORF6/7) protein. J. Virol. *68*, 5027-5035. [Medline]

Hernandez-Alcoceba, R., Pihalja, M., Wicha, M.S., and Clarke, M.F. (2000). A novel, conditionally replicative adenovirus for the treatment of breast cancer that allows controlled replication of E1a-deleted adenoviral vectors. Hum. Gene Ther. *11*, 2009-2024. [Medline]

Howe, J.A. and Bayley, S.T. (1992). Effects of Ad5 E1A mutant viruses on the cell cycle in relation to the binding of cellular proteins including the retinoblastoma protein and cyclin A. Virology *186*, 15-24. [Medline]

Howe, J.A., Mymryk, J.S., Egan, C., Branton, P.E., and Bayley, S.T. (1990). Retinoblastoma growth suppressor and a 300-kDa protein appear to regulate cellular DNA synthesis. Proc. Natl. Acad. Sci. USA *87*, 5883-5887. [Medline]

Johnson, D.G., Ohtani, K., and Nevins, J.R. (1994). Autoregulatory control of E2F1 expression in response to positive and negative regulators of cell cycle progression. Genes Dev. *8*, 1514-1525. [Medline]

Johnson, D.G. and Schneider-Broussard, R. (1998). Role of E2F in cell cycle control and cancer. Front. Biosci. *3*, d447-d448. [Medline]

Jones, N. and Shenk, T. (1979). An adenovirus type 5 early gene function regulates expression of other early viral genes. Proc. Natl. Acad. Sci. USA *76*, 3665-3669. [Medline]

Khuri, F.R., Nemunaitis, J., Ganly, I., Arseneau, J., Tannock, I.F., Romel, L., Gore, M., Ironside, J., MacDougall, R.H., and Heise, C. *et al.* (2000). a controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. Nat. Med. *6*, 879-885. [Medline]

Kirn, D. (2001). Clinical research results with dl1520 (Onyx-015), a replication- selective adenovirus for the treatment of cancer: what have we learned?. Gene Ther. *8*, 89-98. [Medline]

Kirn, D., Martuza, R.L., and Zwiebel, J. (2001). Replication-selective virotherapy for cancer: Biological principles, risk management and future directions. Nat. Med. *7*, 781-787. [Medline]

Kleinberger, T. and Shenk, T. (1993). Adenovirus E4orf4 protein binds to protein phosphatase 2A, and the complex down regulates E1A-enhanced junB transcription. J. Virol. *67*, 7556-7560. [Medline]

Kramm, C.M., Chase, M., Herrlinger, U., Jacobs, A., Pechan, P.A., Rainov, N.G., Sena-Esteves, M., Aghi, M., Barnett, F.H., Chiocca, E.A., and Breakefield, X.O. (1997). Therapeutic efficiency and safety of a second-generation replication- conditional HSV1 vector for brain tumor gene therapy. Hum. Gene Ther. *8*, 2057-2068. [Medline]

Kurihara, T., Brough, D.E., Kovesdi, I., and Kufe, D.W. (2000). Selectivity of a replication-competent adenovirus for human breast carcinoma cells expressing the MUC1 antigen. J. Clin. Invest. *106*, 763-771. [Medline]

Larminie, C.G., Alzuherri, H.M., Cairns, C.A., McLees, A., and White, R.J. (1998). Transcription by RNA polymerases I and III: a potential link between cell growth, protein synthesis and the retinoblastoma protein. J. Mol. Med. *76*, 94-103. [Medline]

Leng, X., Connell-Crowley, L., Goodrich, D., and Harper, J.W. (1997). S-Phase entry upon ectopic expression of G1 cyclin-dependent kinases in the absence of retinoblastoma protein phosphorylation. Curr. Biol. *7*, 709-712. [Medline] [Summary] [Full Text]

Lowe, S.W. and Ruley, H.E. (1993). Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. Genes Dev. *7*, 535-545. [Medline]

McCormick, F. (2001). Cancer gene therapy: fringe or cutting edge?. Nat. Reviews Cancer *1*, 130-141. [Medline]

Mineta, T., Rabkin, S.D., and Martuza, R.L. (1994). Treatment of malignant gliomas using ganciclovir-hypersensitive, ribonucleotide reductase-deficient herpes simplex viral mutant. Cancer Res. *54*, 3963-3966. [Medline]

Mineta, T., Rabkin, S.D., Yazaki, T., Hunter, W.D., and Martuza, R.L. (1995). Attenuated multi-mutated herpes simplex virus-1 for the treatment of malignant gliomas. Nat. Med. *1*, 938-943. [Medline]

Moore, M., Horikoshi, N., and Shenk, T. (1996). Oncogenic potential of the adenovirus E4orf6 protein. Proc. Natl. Acad. Sci. USA *93*, 11295-11301. [Medline]

Moran, E. (1993). DNA tumor virus transforming proteins and the cell cycle. Curr. Opin. Genet. Dev. *3*, 63-70. [Medline]

Muller, H. and Helin, K. (2000). The E2F transcription factors: key regulators of cell proliferation. Biochim. Biophys. Acta *1470*, M1-M12. [Medline]

Muller, U., Kleinberger, T., and Shenk, T. (1992). Adenovirus E4orf4 protein reduces phosphorylation of c-Fos and E1A proteins while simultaneously reducing the level of AP-1. J. Virol. *66*, 5867-5878. [Medline]

Nakamura, H., Mullen, J.T., Chandrasekhar, S., Pawlik, T.M., Yoon, S.S., and Tanabe, K.K. (2001). Multimodality therapy with a replication-conditional herpes simplex virus 1 mutant that expresses yeast cytosine deaminase for intratumoral conversion of 5-fluorocytosine to 5-fluorouracil. Cancer Res. *61*, 5447-5452. [Medline]

Neill, S.D. and Nevins, J.R. (1991). Genetic analysis of the adenovirus E4 6/7 trans activator: interaction with E2F and induction of a stable DNA-protein complex are critical for activity. J. Virol. *65*, 5364-5373. [Medline]

Neill, S.D., Hemstrom, C., Virtanen, A., and Nevins, J.R. (1990). An adenovirus E4 gene product trans-activates E2 transcription and stimulates stable E2F binding through a direct association with E2F. Proc. Natl. Acad. Sci. USA *87*, 2008-2012. [Medline]

Nemunaitis, J., Ganly, I., Khuri, F., Arseneau, J., Kuhn, J., McCarty, T., Landers, S., Maples, P., Romel, L., and Randlev, B. *et al.* (2000). Selective replication and oncolysis in p53 mutant tumors with

ONYX-015, an E1B–55kD gene-deleted adenovirus, in patients with advanced head and neck cancer: a phase II trial. Cancer Res. *60*, 6359-6366. [Medline]

Nemunaitis, J., Cunningham, C., Buchanan, A., Blackburn, A., Edelman, G., Maples, P., Netto, G., Tong, A., Randlev, B., Olson, S., and Kirn, D. (2001). Intravenous infusion of a replication-selective adenovirus (ONYX-015) in cancer patients: safety, feasibility and biological activity. Gene Ther. *8*, 746-759. [Medline]

Nemunaitis, J., Khuri, F., Ganly, I., Arseneau, J., Posner, M., Vokes, E., Kuhn, J., McCarty, T., Landers, S., and Blackburn, A. *et al.* (2001). Phase II trial of intratumoral administration of ONYX-015, a replication-selective adenovirus, in patients with refractory head and neck cancer. J. Clin. Oncol. *19*, 289-298. [Medline]

Neuman, E., Flemington, E.K., Sellers, W.R., and Kaelin, W.G.Jr. (1994). Transcription of the E2F–1 gene is rendered cell cycle dependent by E2F DNA-binding sites within its promoter. Mol. Cell. Biol. *14*, 6607-6615. [Medline]

Nevels, M., Rubenwolf, S., Spruss, T., Wolf, H., and Dobner, T. (1997). The adenovirus E4orf6 protein can promote E1A/E1B-induced focus formation by interfering with p53 tumor suppressor function. Proc. Natl. Acad. Sci. USA *94*, 1206-1211. [Medline]

O'Connor, R.J. and Hearing, P. (1994). Mutually exclusive interaction of the adenovirus E4–6/7 protein and the retinoblastoma gene product with internal domains of E2F–1 and DP-1. J. Virol. *68*, 6848-6862. [Medline]

Obert, S., O'Connor, R.J., Schmid, S., and Hearing, P. (1994). The adenovirus E4–6/7 protein transactivates the E2 promoter by inducing dimerization of a heteromeric E2F complex. Mol. Cell. Biol. *14*, 1333-1346. [Medline]

Parr, M.J., Manome, Y., Tanaka, T., Wen, P., Kufe, D.W., Kaelin, W.G.Jr. , and Fine, H.A. (1997). Tumor-selective transgene expression in vivo mediated by an E2F- responsive adenoviral vector. Nat. Med. *3*, 1145-1149. [Medline]

Querido, E., Marcellus, R.C., Lai, A., Charbonneau, R., Teodoro, J.G., Ketner, G., and Branton, P.E. (1997). Regulation of p53 levels by the E1B 55-kilodalton protein and E4orf6 in adenovirus-infected cells. J. Virol. *71*, 3788-3798. [Medline]

Ramachandra, M., Rahman, A., Zou, A., Vaillancourt, M., Howe, J.A., Antelman, D., Sugarman, B., Demers, G.W., Engler, H., Johnson, D., and Shabram, P. (2001). Re-engineering adenovirus regulatory pathways to enhance oncolytic specificity and efficacy. Nat. Biotechnol. *19*, 1035-1041. [Medline]

Rao, R.N. (1996). Targets for cancer therapy in the cell cycle pathway. Curr. Opin. Oncol. *8*, 516-524. [Medline]

Raychaudhuri, P., Bagchi, S., Neill, S.D., and Nevins, J.R. (1990). Activation of the E2F transcription factor in adenovirus-infected cells involves E1A-dependent stimulation of DNA-binding activity and induction of cooperative binding mediated by an E4 gene product. J. Virol. *64*, 2702-2710. [Medline]

Reichel, R., Neill, S.D., Kovesdi, I., Simon, M.C., Raychaudhuri, P., and Nevins, J.R. (1989). The adenovirus E4 gene, in addition to the E1A gene, is important for trans-activation of E2 transcription and for E2F activation. J. Virol. *63*, 3643-3650. [Medline]

Ries, S.J., Brandts, C.H., Chung, A.S., Biederer, C.H., Hann, B.C., Lipner, E.M., McCormick, F., and Korn, W.M. (2000). Loss of p14ARF in tumor cells facilitates replication of the adenovirus mutant dl1520 (ONYX-015). Nat. Med. *6*, 1128-1133. [Medline]

Rodriguez, R., Schuur, E.R., Lim, H.Y., Henderson, G.A., Simons, J.W., and Henderson, D.R. (1997). Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. Cancer Res. *57*, 2559-2563. [Medline]

Schaley, J., O'Connor, R.J., Taylor, L.J., Bar-Sagi, D., and Hearing, P. (2000). Induction of the cellular E2F–1 promoter by the adenovirus E4–6/7 protein. J. Virol. *74*, 2084-2093. [Medline]

Shen, Y., Kitzes, G., Nye, J.A., Fattaey, A., and Hermiston, T. (2001). Analyses of single-amino-acid substitution mutants of adenovirus type 5 E1B–55K protein. J. Virol. *75*, 4297-4307. [Medline]

Sherr, C.J. (2000). The Pezcoller lecture: cancer cell cycles revisited. Cancer Res. *60*, 3689-3695. [Medline]

Steegenga, W.T., Riteco, N., Jochemsen, A.G., Fallaux, F.J., and Bos, J.L. (1998). The large E1B protein together with the E4orf6 protein target p53 for active degradation in adenovirus infected cells. Oncogene *16*, 349-357. [Medline]

Teodoro, J.G. and Branton, P.E. (1997). Regulation of p53-dependent apoptosis, transcriptional repression, and cell transformation by phosphorylation of the 55-kilodalton E1B protein of human adenovirus type 5. J. Virol. *71*, 3620-3627. [Medline]

Teodoro, J.G., Halliday, T., Whalen, S.G., Takayesu, D., Graham, F.L., and Branton, P.E. (1994). Phosphorylation at the carboxy terminus of the 55-kilodalton adenovirus type 5 E1B protein regulates transforming activity. J. Virol. *68*, 776-786. [Medline]

Wang, H.G., Draetta, G., and Moran, E. (1991). E1A induces phosphorylation of the retinoblastoma protein independently of direct physical association between the E1A and retinoblastoma products. Mol. Cell. Biol. *11*, 4253-4265. [Medline]

Wang, H.G., Rikitake, Y., Carter, M.C., Yaciuk, P., Abraham, S.E., Zerler, B., and Moran, E. (1993). Identification of specific adenovirus E1A N-terminal residues critical to the binding of cellular proteins and to the control of cell growth. J. Virol. *67*, 476-488. [Medline]

Weinberg, R.A. (1995). The retinoblastoma protein and cell cycle control. Cell *81*, 323-330. [Medline]

Whalen, S.G., Marcellus, R.C., Whalen, A., Ahn, N.G., Ricciardi, R.P., and Branton, P.E. (1997). Phosphorylation within the transactivation domain of adenovirus E1A protein by mitogen-activated protein kinase regulates expression of early region 4. J. Virol. *71*, 3545-3553. [Medline]

White, E. (1995). Regulation of p53-dependent apoptosis by E1A and E1B. Curr. Top. Microbiol. Immunol. *199*, 34-58. [Medline]

Whyte, P., Williamson, N.M., and Harlow, E. (1989). Cellular targets for transformation by the adenovirus E1A proteins. Cell *56*, 67-75. [Medline]

Yang, C.T., You, L., Uematsu, K., Yeh, C.C., McCormick, F., and Jablons, D.M. (2001). p14(ARF) modulates the cytolytic effect of ONYX-015 in mesothelioma cells with wild-type p53. Cancer Res. *61*, 5959-5963. [Medline]

Yang, Y., Nunes, F.A., Berencsi, K., Furth, E.E., Gonczol, E., and Wilson, J.M. (1994). Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. Proc. Natl. Acad. Sci. USA *91*, 4407-4411. [Medline]

Yew, P.R. and Berk, A.J. (1992). Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. Nature *357*, 82-85. [Medline]

Yew, P.R., Liu, X., and Berk, A.J. (1994). Adenovirus E1B oncoprotein tethers a transcriptional repression domain to p53. Genes Dev. *8*, 190-202. [Medline]

Yu, D.C., Chen, Y., Seng, M., Dilley, J., and Henderson, D.R. (1999). The addition of adenovirus type 5 region E3 enables calydon virus 787 to eliminate distant prostate tumor xenografts. Cancer Res. *59*, 4200-4203. [Medline]

Yu, D.C., Sakamoto, G.T., and Henderson, D.R. (1999). Identification of the transcriptional regulatory sequences of human kallikrein 2 and their use in the construction of calydon virus *764*, an attenuated replication competent adenovirus for prostate cancer therapy. Cancer Res. *59*, 1498-1504. [Medline]

Footnotes

4 Present address: Exelixis, South San Francisco, California 94083

6 Present address: Berlex Laboratories, Inc., Richmond, California 94804

7 Present address: Cerus Corporation, Concord, California 94520

5 Present address: ProteinPlanet, San Francisco, California 94115

Received: March 4, 2002
Revised: April 23, 2002

This article has been cited by the following articles:

- **The RB and p53 pathways in cancer**
  Charles J. Sherr and Frank McCormick
  Cancer Cell, 2002, 2:2:103-112
  [Summary] [Full Text] [PDF]
- **(Re-)Engineering tumor cell-selective replicating adenoviruses: A step in the right direction toward systemic therapy for metastatic disease**
  Thomas W. Dubensky
  Cancer Cell, 2002, 1:4:307-309
  [Summary] [Full Text] [PDF]